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(54) Title: CONDITIONALLY IMMORTALIZED GERM CELL LINES

(57) Abstract

The invention relates to immortalized and conditionally immortalized germ cell lines, and in particular to immortalized and conditionally immortalized testicular cell lines. Seminiferous-tubule-like structures can be produced *in vitro* using the immortalized cell lines of the present invention. Methods of producing such cell lines are also provided as well as methods for the *in vitro* production of proteins expressed by these cell lines. In a further aspect, the present invention provides methods of controlling the proliferation and differentiation of immortalized germ cells for a variety of purposes, including *in vitro* fertilization and the production of transgenic mice.

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# CONDITIONALLY IMMORTALIZED GERM CELL LINES

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## BACKGROUND OF THE INVENTION

The present invention generally relates to immortalized and conditionally immortalized cell lines, each associated with spermatogenesis, and uses for such cell lines.

Mature s[illegible] cells in animals originate from germ cells that have undergone a process referred to as spermatogenesis. This process takes place within a specialized microenvironment of the seminiferous or sperm-producing tubule located in the testis.

In maturing to sperm cells, germ cells progress through several stages of differentiation during this process. For example, mouse germ cells proceed through twelve stages of meiotic differentiation, including primitive type A, type A and type B spermatogonia, preleptotene, leptotene, zygotene and pachytene spermatocytes (generally referred to as primary spermatocytes), secondary spermatocytes and round spermatids. Further differentiation to mature sperm cells is believed to take place during the journey from the seminiferous tubule to the epididymis in the *in vivo* environment. Little is known, however, about the factors and conditions that regulate the differentiation of germ cells throughout the spermatogenesis process.

It is known that germ cells are influenced by various somatic cells within the specializ d microenvironment. The somatic cells that influence germ cells during spermatogenesis are referred to as Sertoli, peritubular and Leydig cells.

Sertoli cells are the only somatic cells located within the lumen of the seminiferous tubule. They are responsible for providing all nutrients and protein factors needed for germ cell differentiation. Such protein factors include, for example, androgen binding protein, transferrin, ceruloplasmin, clusterin, testibumin, plasminogen activator, $\alpha$-2 macroglobulin, inhibins, growth factors, type IV collagen, laminin and others. Sertoli cells respond to the hormone FSH.

Myeloid peritubular cells constitute the walls of the seminiferous tubule and are mostly responsible for secreting extracellular matrix molecules. Such cells have been found to secrete fibronectin and types I and IV collagen. Peritubular cells act cooperatively with Sertoli cells in the formation and deposition of the extracellular matrix molecules, but the extent of such interaction is not well understood.

Leydig cells are the single most important steroidogenic cells in the testis. They metabolize steroids and are believed to be the principal sites of testosterone production in the testis. Sertoli cells also metabolize certain testicular steroids but to a lesser extent than Leydig cells. Leydig cells respond to hormonal signals, such as LH and hCG, by activating the steroid pathway which leads to the production of testosterone and other steroids. Such steroids have been implicated in the process of meiosis and differentiation of germ cells. However, whether these effects ar

exerted directly on th germ cells or indirectly through their effects on the somatic cells is not clear.

Although it is known that somatic cells, extracellular matrix molecules, hormones and steroids influence the differentiation of germ cells, the exact nature of these interactions and of the molecules involved are not known. It appears that spermatogenesis results from the control and regulation of complex and diverse cellular interactions and communications.

An understanding of the ability of germ cells to switch from mitotic proliferation to terminal differentiation would be helpful for the understanding and treatment of cancer since tumors are known for their continued cell proliferation. In addition, the study of germ cells _in vitro_ would enable the study, diagnosis and treatment of infertility, and would be useful in evaluating potential gene therapy strategies to correct genetic defects.

Germ cells that have differentiated to mature sperm under controlled conditions would be useful for _in vitro_ fertilization or to produce transgenic animals, such as transgenic mice, by _in vitro_ fertilization.

Thus, a need exists for both immortalized or permanent cell lines, and conditionally immortalized cell lines capable of switching between proliferation and differentiation under appropriate conditions. Such cell lines can be used as _in vitro_ models of the somatic and germ cell lines to study spermatogenesis and for _in vitro_ fertilization as well as other purposes. Attempts at long term cultures of primary somatic cells and mixed primary somatic and germ cells have been largely unsuccessful. Such attempts have generally involved the _in vitro_ cultures of mixed cell types that seldom

exceeded fifteen days. To date, no permanent germ cell lines have been establish d. The present invention satisfies the ne d for such cell lines and provides related advantages as well.

## SUMMARY OF THE INVENTION

The present invention provides a set of germ cell lines which includes both immortalized germ cell lines, and conditionally immortalized germ cell lines. More particularly, the invention provides a set of murine testicular cell lines which includes both immortalized and conditionally immortalized murine testicular germ cell lines. These murine germ cell lines are immortalized or conditionally immortalized at various stages of sperm cell differentiation. The immortalized germ cell lines are permanent cell lines which do not proceed through meoisis in vitro, whereas the conditionally immortalized cell lines are capable of switching between proliferation and differentiation under appropriate conditions.

The invention further relates to a method of controlling the proliferation or differentiation of immortalized germ cell lines which contain a gene encoding for an immortalizing molecule by activating or deactivating the expression of the immortalizing molecule. Such an immortalized germ cell could be induced to differentiate into a mature sperm cell in vitro, which in turn can be used for in vitro fertilization.

In another aspect, the invention also relates to the in vitro production of proteins, such as a testicular isoform of cytochrome c and LDH-C, which ar expressed by certain of these immortaliz d or conditionally immortalized germ cell lines.

In a further aspect, the present invention relates to immortaliz d somatic cell lines derived from mammalian testis capable of being cultured *in vitro* for at least one month or longer. Such somatic cell lines include Sertoli, myeloid peritubular and Leydig cell lines.

The immortalized somatic cell lines can be combined with the immortalized germ cell line to produce seminiferous tubule-like structures *in vitro*. The structures are useful as *in vitro* models for the study of spermatogenesis and cell-cell and cell-matrix interaction phenomena and to identify molecules involved in the process of tissue morphogenesis. The structures can be detected by labeling at least one cell line with a detectable marker, such as a fluorescent marker.

In another aspect of the present invention, the immortalized germ cell lines can be used to produce transgenic animals, such as transgenic mice.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows an immunocytochemical analysis of germ cell lines GC-2spd(ts) and GC-3spd(ts) grown at 37°C (Figure 1A), and grown at 32°C (Figure 1B).

Figure 2A shows periodic acid-Schiff staining of the acrosomal granule in GC-2spd(ts) round spermatids; Figure 2B shows immunocytochemical staining of GC-2spd(ts) of the acrosomal granule in GC-2spd(ts) round spermatids with monoclonal antibody HS-63. Figure 2C shows an electron micrograph of the acrosomal granule.

Figure 3 shows flow cytometric analysis of GC-2spd(ts) cell lin grown at 37°C for 16 generations (Figure 3A), 24 generations (Figure 3B), 30 generations

(Figure 3C), compared with control mous testis (Figure 3D).

## DETAILED DESCRIPTION OF THE INVENTION

The present invention provides immortalized and conditionally immortalized non-tumorigenic germ cell lines. Prior to the present invention, those skilled in the art have for many years unsuccessfully attempted to produce a germ cell line capable of being cultured *in vitro* for an indefinite period of time. Such immortalized and conditionally immortalized germ cell lines are useful as an *in vitro* model to study spermatogenesis. They are also useful as a model to identify and manipulate the factors that control germ cell proliferation and differentiation. In addition, *in vitro* germ cell lines are induced to differentiate into mature sperm cells, which in turn are used for *in vitro* fertilization to produce transgenic animals, such as transgenic mice.

As used herein the term "immortalized cell line" refers to a cell line in which a foreign gene encoding for an immortalizing molecule is integrated into a naturally occurring cell line such that the line can then be cultured *in vitro* for an indefinite period of time. As used herein, "immortalized or permanent cell line" refers to cells that can be cultured *in vitro* for at least one month. Such immortalized or permanent cell lines are viable whether continuously cultured *in vitro* or stored according to methods known in the art. As used herein the term "immortalized germ cell line" refers to a germ cell line which has been immortalized but which is non-tumorigenic and non-malignant.

As used herein the term "conditionally immortalized cell line" refers to an immortalized cell

line in which th immortalizing molecule can be activated or inactivated under appropriate conditions. When the imm rtalizing molecule is activated, the cell line proceeds with mitotic proliferation, and when the immortalizing molecule in inactivated, the cell line proceeds to differentiate. A conditionally immortalized cell line can be constructed in a number of ways. A foreign gene encoding a temperature-sensitive immortalizing molecule, such as the polyoma large T antigen, can be integrated in the genome of a cell. The resulting cell line can be grown at temperatures at which the immortalizing molecule is active, or at which the molecule becomes inactive, such as between about 38°C and 40°C. In another embodiment, an immortalizing molecule can be operably linked to an inducible promoter that drives the expression of the immortalizing molecule. Alteratively, a cell line can be cotransformed with a gene encodin an immortalizing molecule and a gene encoding a ı ecule which binds with and inactivates the immortalizing molecule under certain conditions. For example, a cell line can be transformed with an immortalizing large T antigen, and a temperature-sensitive antiproliferative protein such as p53 capable of inactivating the large T antigen in the cell. As used herein, the term "conditionally immortalized germ cell line" refers to a germ cell line which has been conditionally immortalized but which is non-tumorigenic and non-malignant.

As used herein the term "antiproliferative protein" refers to tumor suppressor gene products such as cellular phosphoprotein p53 or Rb which have been found to complex with the transforming proteins of DNA tumor viruses in transformed cells. The p53 protein has been identified as associated with the large T antigen in SV-40 transformed cells (Lane et al. Nature (London) 278: 261-263 (1979), and Linzer et al., Cell 17:43-52 (1979)),

th E1b 55 kDa protein of adenovirus (Sarnow et al. Cell 28:387-394 (1982)), and the E6 prot in of papillomavirus (Wern ss t al. Science 248: 76-79 (1990)). Exogenous wild type (wt) p53 has been shown to have antitransforming activity (Finlay et al. Mol Cell Biol 8: 531-539 (1988)) when co-transfected with transforming proteins. In addition, wtp53 was found in mutated form, in reduced amounts, or to be absent in a wide variety of naturally occuring human tumors.

As used herein, the term "crisis" refers to a primary cell culture which is undergoing a number of cell divisions during which most of the secondary cells die and disintegrate; the remaining cells have the potential to give rise to an established cell line. As used herein the term "precrisis" refers to the state of a primary cell culture before crisis.

According to the present invention, a germ cell line is immortalized by integrating a plasmid containing a gene encoding for an immortalizing molecule into the chromosome of a naturally occurring germ cell. For example, a germ cell from the testis of a male animal can be transfected with the plasmid, pSV3-neo (ATCC No. 37150), which contains a gene encoding for the SV40 large T antigen. The SV40 large T antigen is known to be an immortalizing molecule. Other plasmids containing genes encoding for large T antigens or other immortalizing molecules having immortalizing properties similar to the large T antigen, such as E,A (adenovirus), c-myc, c-jun, h-ras and v-scr, which are all well known in the art, can also be used. The SV40 large T antigen, a nuclear factor that normally regulates SV40 viral replication, is used to immortalize the germ cells at a particular stage of differentiation by virtue of its replicative functions. More particularly, the large T antigen locks the cells at the d velopmental and gene expression stage of early

meiosis, whil forcing the cells to cycle once again by mitosis. Other large T antigens useful in the present invention include, for example, polyoma virus large T antigens and temperature-sensitive polyoma large T antigens, as described in Linder et al., Exp. Cell Res. 191:1-7 (1990) and J. Chou, PNAS USA 75:1403-1413 (1978).

The methods for cultivating germ cell lines for the purpose of immortalizing them differ from previous attempts at the long term culture of non-tumorigenic germ cells is that the germ cells were grown in the presence of a monolayer c. the somatic testicular cells prior to immortalizing with the large T antigen. Thus, the germ cells were cultured for a few days in a seminiferous tubule-like environment prior to immortalization.

Germ cells can be immortalized at defined stages of differentiation including, for example, primitive type A, · pe A or B spermatogonia, primary spermatocytes or secondary spermatocytes. Germ cells can be pre-selected at a desired differentiation stage before an immortalizing plasmid is incorporated into the genome. For example, germ cells taken from a testis corresponding to the different stages of differentiation can be separated using unit gravity sedimentation procedures, which are well known in the art. For example, the STA-PUT™ sedimentation procedure (Johns Scientific, Toronto, Canada) can be used, which is described in Romrell et al., Dev. Biol. 49:119-131 (1976). The type of immortalized germ cell line to be obtained therefore depends on the amount of differentiation a germ cell has undergone at the time the cell is extracted from a male animal.

An immortalized or conditionally immortalized germ cell line can be characterized according to its stage of differentiation by detailed microscopic and

electron microscopic examination. The presence or absenc of stage-specific protein markers can also b used. For example, a germ cell line can be characterized as a primary spermatocyte by its expression of testis-specific lactate dehydrogenase (LDH-C) and the testicular isoform of cytochrome c (cytochrome $c_t$). The immortalized germ cell line designated as GC-1 is believed to be in a transition stage between spermatogonia and the primary spermatocyte stage. Studies indicate that the GC-1 expresses cytochrome $c_t$ and LDH-C. The conditionally immortalized germ cell lines designated GC-2spd(ts) and GC-3spd(ts) can be characterized as in the secondary spermatocyte stage. At the permissive temperature of 37°C, LDH-C production was enhanced, although cytochrome $c_t$ production is reduced. Thus, a further aspect of the present invention is a method of producing such isoproteins <u>in vitro</u> by culturing immortalized or conditionally immortalized germ cells expressing such isoproteins, and harvesting the isoproteins by methods known in the art such as described, for example, in Goldberg, <u>J. Biol. Chem</u>. 247:2044-2048 (1972).

In another aspect of the present invention, conditionally immortalized germ cell lines are constructed in which the immortalizing molecule can be modulated under certain conditions to allow the cell lines to either proliferate or undergo differentiation <u>in vitro</u>. When the immortalizing molecule is activated, the cell line proceeds with mitosis; when the immortalizing molecule is inactivated, the cell line proceeds with differentiation. Constructing conditionally immortalized germ cell lines can be accomplished for example, by transfecting the primary germ cell with a gene encoding a temperature-sensitive immortalizing molecule. When the conditionally immortalized cell line is kept at permissive temperatur s, the expressed immortalizing molecule is active, and the cell lines

proliferate. At a non-permissive temperature, the immortalizing molecule is inactivated, and the cells can proceed through differentiation. In another embodiment, an immortalizing molecule is operably linked to an inducible promoter that drives the expression of the immortalizing molecule.

Alternatively, activation and deactivation is controlled by a gene encoding a molecule which binds to and inactivates the immortalizing molecule inside the cell under certain conditions. Such cell lines are constructed by cotransfecting both a gene encoding for an immortalizing molecule and a gene encoding for an antiproliferative molecule capable of binding the immortalizing molecule under certain conditions. For example, primary germ cell populations enriched in secondary spermatocytes were cotransfected with a gene encoding an immortalizing molecule and a gene encoding an antiproliferative molecules capable of binding the immortalizing molecule. In one preferred embodiment, primary mouse germ cells were cotransfected with the SV40 Large T (LTAg) antigen gene and the gene encoding for a temperature-sensitive (ts) mutant of p53, to obtain cell lines in which both moelcules were expresssed. Two secondary spermatocyte cell lines, GC-2spd(ts) and GC-3spd(ts) were established in which an excess of p53 was able to bind LTAg at the permissive temperature, thus reducing the proliferative effects of LTAg. One of these cell lines in particular, GC-2spd(ts), was shown to be capable of differentiating and undergoing meoisis <u>in vitro</u>. Differentiation was characterized by immunocytochemical and morphological observation, as described in detail in Example V.

In another aspect, the present invention relates to m thods of controlling the proliferation or differentiation of a conditionally immortalized germ cell

lin by activating or deactivating th expressed immortalizing mol cule. Prolif ration of an immortalized germ cell can b facilitated by the presence of the immoralizing molecule, while differentiation can be facilitated by the inactivation of the molecule. Methods include the use of a temperature-sensitive immortalizing molecule, such as temperature sensitive large T antigens, which are well-known in the art. Methods also include the linkage of a gene encoding an immortalizing molecule to an inducible promoter which drives the expression of the immortalizing molecule, or the cotransfection of a primary cell with a gene encoding the immortalizing molecule and with a gene encoding a molecule capable of inactivating the immortalizing molecule under certain conditions.

The present invention also relates to immortalized somatic cell lines derived from non-tumorigenic testicular cells of an animal, particularly Sertoli, peritubular and Leydig cell lines, which are capable of being cultured _in vitro_ for at least one month and preferably indefinitely.

A few somatic cell lines with characteristics of Sertoli, peritubular and Leydig cells have been previously cultured _in vitro_. For example, cultured Leydig-like cells are described in Mather, _Biol. Reprod_. 23:243-252 (1980) and are available under the ATCC Nos. CRL 1714 and CRL 1715, while Yasumura et al., _Science_ 154:1186 (1966) describes Leydig-like tumor cell lines (ATCC No. CCL 83). Cultured peritubular-like cell lines are described in Mather et al., _J. Ultrastructural Res_. 87:263-274 (1984). However, these known cell lines differ from the somatic cell lines of the present invention in several ways. Th known somatic cell lines are mostly derived from spontaneous tumors or other abnormal tissues, whereas the cell lines of the present

invention display normal morphology and are considered non-tumorigenic.

The present cell lines have been immortalized or conditionally immortalized from cells of the same developmental stage using a consistent and minimal genetic change, that is, introduction and stable integration of the pSV3-neo plasmid, and expression of the SV40 large T antigen gene, and for some cell lines the p53 gene as well. Previous attempts at long term cultures, on the other hand, have involved combining at least two cell types of the somatic cell environment or co-cultures of Sertoli and germ cells as the primary cultures.

Furthermore, the viability of these known primary co-cultures seldom exceeds fifteen days, whereas the immortalized somatic cell lines of the present invention have been cultured for more than 2 years. The known long-term cultures of such somatic cells have been derived generally from tumor cells compared with the immortalized non-tumorigenic cell lines of the present invention.

When cells from each of the immortalized somatic and germ cell lines are plated together, they reaggregate to form <u>in vitro</u> seminiferous tubule-like structures. The germ cells appear in the center of these formations, while Sertoli, peritubular and Leydig cells establish borders between these germ cell cores, similar to the structures produced by non-tumorigenic, freshly isolated testicular cells. Thus, immortalized germ cells can be cultured in the presence of fresh or immortalized somatic cells within the <u>in vitro</u> seminiferous tubule-like environment. Conversely, immortalized somatic cells can be used to grow and induce the differentiation of non-immortalized germ cells.

The formation of the tubule-like structures can be visually monitored under microscopic observation as well as aided by detectabl markers, such as fluorescent dyes. Cells from at least one immortalized cell line can be labeled with a fluorescent dye, such as DiI (D-282) or DiO (D-275) (Molecular Probes, Eugene, OR), or other known labels or methods as described, for example, in Honig et al., J. Cell Biol. 103:171-187 (1980). Other dyes that can be used include 1% trypan blue, trypan red or india ink in $H_2O$, 0.025-0.25% methylene blue, Tanus green B or neutral red.

The immortalized and conditionally immortalized cell lines of the present invention can be used for a variety of purposes. For example, paracrine and autocrine factors responsible for germ cell differentiation, nutrient requirements, steroidogenic pathways and conditions that will allow non-tumorigenic germ cells to be cultured *in vitro* can be studied using the tubule-like structures as an *in vitro* model. In addition, immortalized germ cells that have differentiated to the spermatid stage or beyond, thus completing the second meiotic division, can be used to fertilize oocytes *in vitro* by nuclear transfer or cell fusion techniques. Such techniques are well known in the art such as described, for example, in Palmiter et al., Ann. Rev. Genet. 20:465-99 (1986). If differentiation continues to the mature sperm stage, the conventional *in vitro* fertilization procedures can be used. Such procedures can be used to produce transgenic mice, for example. Additionally, germ cells from a prized animal can be immortalized at a particular stage, frozen or cultured for an indefinite period and later used for *in vitro* fertilization. Thus, the present invention can be used for breeding certain desirable characteristics.

Th following examples are intended to illustrate but not limit the following invention.

## EXAMPLE I

## Immortalization of Cells From Immature Mouse Testis

Testes from four 10-day old Balb/C mice were collected aseptically in serum-free CMRL-1066 culture medium (Gibco, Bethesda, MD), rinsed in 0.1 M phosphate buffered saline (PBS) and treated with collagenase (1 mg/ml in PBS) (Boehinger-Mannheim) for 15 minutes at room temperature. The medium was replaced by PBS containing DNase (10 ug/ml)(Sigma Chemical Co., St. Louis, MO) and the mixture pipetted up and down several times until a cell suspension was obtained. Cells were washed in PBS, then layered onto a discontinuous Percoll (Pharmacia, Piscataway, NJ) gradient composed of 4 layers with densities of 1.055, 1.045, 1.035 and 1.025, respectively, as described in Schumacher et al., FEBS LETT. 91:333-38 (1978). Centrifugation was carried out at 200 x g for 20 minutes in a bench-top centrifuge. Cells were distributed into 3 bands, corresponding to the 3 interphases of the Percoll gradient. Based on their morphology, adherence potential and growth characteristics, band A (density = 1.030) was enriched in spermatogonia and primary spermatocytes, band B (density = 1.040) in Sertoli and peritubular cells, and band C (density = 1.050) was enriched in Leydig and endothelial cells, both obtained from the same testes. Cells of each band were cultivated separately in tissue culture flasks (Falcon). Cell survival was optimal (10 days for spermatogonia, more than 2C days for somatic cells) in CMRL-1066 medium enriched with 80 μg/ml insulin, 3 μg/ml transferrin, 80 μg/ml ascorbic acid and 13% inactivated fetal calf serum.

Cells of bands B and C were cultivated for 2 w eks until monolayers were formed. Th se bands, that contain all four cell types although in different relative proportions, reconstituted tubuli-like structures _in vitro_, with germ cells always homing to the center of these formations. These monolayers (in 25 $cm^2$ flasks) were transfected by the calcium phosphate procedure as described in Gorman et al., _Mol. Cell. Biol._ 2:1044-1051 (1982), incorporated herein by reference, with 25 $\mu g$ of the pSV3-neo plasmid (ATCC #37150) that contains the genes coding for the immortalizing SV40 large-T antigen and resistance to neomycin (selection marker). After transfection, cells were given fresh culture medium. Four days later, G418 (geneticin, Gibco) was added at a concentration of 200 $\mu g/ml$ of active substance. At weekly intervals, the selection medium was changed until colonies appeared.

Cell colonies surviving the selection procedure were picked by gentle scrapping and cultivated separately in culture medium. Immortalized colonies were obtained at a frequency of about $10^{-6}$. No colonies survived when parallel cultures were transfected with the non-immortalizing plasmid control, pSV2-neo, (ATCC # 37198) which lacks the SV40 large T antigen. Since the resulting immortalized colonies were not pure, extensive clonings by limiting dilution (4 to 6 single-cell clonings per cell line) were carried out, yielding a total of 49 lines. These cell lines have now been continuously cultured for several generations over a period of 2.5 years and are considered permanent cell lines.

## EXAMPLE II

## Integration of the SV40 Large T Antigen Gene

After trypsinization, cells were washed once in PBS and pelleted by centrifugation. Genomic DNA was recovered from cell pellets by treatment with 1% SDS and 50 μg/ml proteinase K in TE buffer (0.01 Tris, 0.001 M EDTA, pH 7.5). DNA was extracted with phenol and chloroform and recovered by ethanol precipitation according to well known standard methods as described in Sambrook et al., Molecular Cloning: A laboratory manual, Cold Spring Harbor Laboratory Press (N.Y. 1989), incorporated herein by reference. Fifteen μg of genomic DNA and 1 ng of pSV3-neo DNA (positive control) were digested with Bam HI in order to excise the large T antigen gene. The samples containing PG3 peritubular cell DNA, SH2 Sertoli cell DNA, LAB2 Leydig cell DNA and germ cell DNA were electrophoresed in 0.8% agarose gel and transferred onto a nitrocellulose filter. The filter was hybridized with a $^{32}$P-labelled Bam HI fragment of the pSV3-neo.

All the permanent cell lines integrated the SV40 large T antigen gene in their genome as shown by conventional Southern blot analysis of the cellular high molecular weight DNA. A 3.3 kb fragment was detected that corresponds to the large T antigen gene. These results confirm that the immortalizing gene has been integrated in the genome of these cells. In addition, these cells show expression of the large T antigen with the typical nuclear localization, as shown by immunocytochemistry.

## EXAMPLE III

## Characterization of the SV40 Large T Antigen Immortalized Cell Lines

The forty-nine permanent cell lines obtained were characterized further according to their morphology, presence of histochemical and immunohistochemical markers, their lack of growth in soft agar and their ability to reconstitute tubule-like structures when the cells were combined *in vitro*. Table 1 summarizes the results obtained in these characterization studies.

TABLE 1

| | SERTOLI | LEYDIG | PERITUBULAR | GERM CELLS |
|---|---|---|---|---|
| # of clones | 8 | 22 | 16 | 3 |
| growth in soft agar | 1 of 8 | 3 of 22 | 5 of 16 | 0 |
| morphology of non-transformed clones | normal | normal | normal | normal |
| immunohistochemical markers | ND | 3 ß-ol DH; Alkaline phos. | Desmin; Alkaline phos. | Cytochrome $C_t$; |
| Morphogenic potential for tubule-like structures | conserved | conserved | conserved | conserved |

The 49 permanent cell lines have been designated as follows: Sertoli (SC5, SH2, SC1, SF7, SG4, SB7, SC72 and SE121), peritubular (PE8, PG3, PE11, PG52, PB51, PF71, PG121, PH121, PD32, PE102, PB83, PD103, PC111, PD122, PA122 and PH12), Leydig (LJG1, LEC2, LAC11, LCF6, LB32/G4, LB32/E3, LIF8, LB32/B7, LFG6, LFA2, LBC12, LEA7, LEB10, LED2, LFA6, LBB7, LAB2, LHD7, LGC12, LB1ro, LAH7 and LFB3) and Germ cell (GC-1, GC-2 AND GC-3).

A. Test for Malignant Transformation

The use of oncogenes to immortalize cells is known to result occasionally in the malignant transformation of the cells as evidenced by their ability to grow in soft agar and/or in nude mice. To determine whether the immortalized cells had undergone malignant transformation, cell monolayers were first trypsinized, treated with 1 mg/ml collagenase in PBS until a single-cell suspension was obtained and washed several times in PBS. Soft agar cultures were performed following the method of Hamburger and Salmon, Science 197:461 (1977), incorporated herein by reference. Briefly, $1x10^6$ cells were resuspended in warm CMRL-1066 medium fortified as described in Example I and containing 0.3% agar noble (Difco Laboratories, Detroit, MN). The mixture was poured onto an underlayer previously prepared in 60 mm diameter culture dishes. The underlayer was made of 0.6% agar in McCoy's medium 5a completed with 1% sodium pyruvate, 42 µg/ml L-serine, 1% glutamine (200 mM), 0.15% tryptic soy broth, 1% penicillin/strep and 13% inactivated fetal calf serum. Cultures were incubated at 37°C and 5% $CO_2$ for at least one week before assessment of clonal proliferation. Only between 12.5% and 32.5% of the immortalized clones were able to grow in soft agar, i.e., 1 out of 8 Sertoli (SC72), 3 out of 22 Leydig (LFA2, LED2 and LAH7) and 5 out of 16 peritubular (PG52, PB51, PF71, PE102 and PB83) cells, while the majority of the immortalized somatic cell lines and our immortalized germ cell clone (GC-1) had retained a normal growth behavior including contact inhibition. Contact inhibition is a property of non-tumorigenic, non-malignant cells in which the cells proliferate until they come into contact with adjacent cells to establish a monolayer. Malignant cells, on the other hand, will not

arrest at this stage and will continu to grow on top of each other.

B. Electronmicroscopic Analysis

The cells were also analyzed by electronmicroscopy. The cell lines were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, and postfixed in 1% $OsO_4$ followed by saturated thiocarbohydrazide and a second $OsO_4$ treatment. The fixed cells were stained with 2% uranyl acetate and embedded in an Epon 812 mixture (Shell Epon Resin 812, E.V. Roberts & Associates, Palo Alto, CA). Sections were obtained with a Reichert Ultracut E ultramicrotome and viewed with an Hitachi H-600 electron microscope.

Electronmicrographs show distinctive features of Sertoli and peritubular cells. Characteristic features of Sertoli cell line SH2 include lipid and glycogen inclusions and an abundant agranular reticulum as well as numerous spherical and elongated mitochondria exhibiting transverse cristae of orthodox lamellar configuration. Characteristic features of the peritubular cell line PG3 include longitudinal microtubules, abundant microfilaments and secretion and deposition of a collagen-rich extracellular matrix.

Electronmicrographs show incomplete cytokinesis in cultures of the immortalized germ cell line GC-1. At any one time, approximately 5% of the GC-1 cells can be found in this state. This incomplete cytokinesis is not observed with the somatic cell lines, and resembles the in vivo situation where primary spermatocytes undergo meiosis connected by cytoplasmic bridges in a syncytium-like fashion.

C. Immunohistochemical Characterization

To characterize the specific markers expressed by the different immortalized cell types, the immortalized cells were cultured to confluency in Lab-Tek chamber slides (Nunc, Naperville, IL) with complete D-MEM medium and fixed with cold methanol. Antigens were revealed by using an immunoperoxidase technique according to the manufacturer's directions (Vectastain ABC kit, Vector, Burlingame, CA). The primary antibodies used were mouse monoclonal anti-SV40 large T antigen (1:100 in PBS) (Oncogene Science, Manhasset, NY), and mouse monoclonal anti-desmin (1:100 in PBS) (Amersham, Arlington Heights, IL). The rabbit polyclonal and mous monoclonal antibodies against the testis-specific LDH-C isozyme and the rabbit polyclonal antibodies against the testis-specific isoform of cytochrome c (designated cytochrome $c_t$) were supplied by Dr. Erwin Goldberg (Northwestern University, Illinois). Counterstaining was performed with hematoxylin, fast green or neutral red.

Two histochemical markers were detected. The 3ß-hydroxysteroid dehydrogenase (3ß-HDH) assay, an enzyme that converts pregnenolone to progesterone and is characteristic of Leydig cells, was performed according to the method described by Wiebe et al. in Endocrinology 98:505-513 (1976), using dehydroepiandrosterone, ß-NAD and nitro blue tetrazolium. After the reaction was completed, cells were fixed in 4% formalin in PBS, pH 7.2 (buffered formalin) and counterstained with 0.1% neutral red. For detection of total alkaline phosphatase activity, Leydig and peritubular cells were first fixed with buffered formalin, stained with Naphthol As-MX phosphate (Sigma Chemical Company, St. Louis MO) and Fast Violet B salt (Sigma Chemical Co.) by a well known procedure described in Wiebe, Endocrinology 98:505-513 (1976), which is incorporated herein by reference, and

counterstained with hematoxylin according to standard conventional procedures.

Both peritubular and Leydig cells were found positive for alkaline phosphatase. The alkaline phosphatase staining in peritubular cells was preferentially associated with spindle-like growth of th cells and was much weaker when the cells were more spars and appeared with round morphology. The color of the alkaline phosphatase reaction product in peritubular and Leydig cells was consistently different, suggesting that different isozymes are expressed. Preliminary Northern blot results indicate that this may be the case. These results were confirmed by reverse-transcriptase polymerase chain reaction amplification (RT-PCR) of poly A + mRNA extracted from these cell types, using isozyme-specific oligonucleotide primers, as previously described.

Sertoli cells were negative histochemically for alkaline phosphatase as was the germ cell line GC-1. Peritubular cells were selectively stained with a monoclonal antibody against desmin. The murine anti-desmin antibody was obtained from Amersham (Arlington Heights, Illinois) and is described in Anthony et al., Biol. Reprod. 40:811-823 (1989). Similarly, Leydig cells exhibited the characteristic intracytoplasmic dotted staining for the enzyme 3ß-HDH. These cell-type characteristics were preserved when all four immortalized cell lines were plated together and reconstituted tubule-like structures as described in Example IV.

The germ cell line, GC-1, was negative for the markers previously identified in the somatic cell lines. It, however, has been shown to express the testicular isoform of cytochrome c. Initial studies indicate that these cells can also express the testis-specific lactate

dehydrogenase (LDH-C). These isozymes are recognized as being specific for the spermatogenic lineage and expressed from the preleptotene stage of germ cell differentiation onward. The GC-1 line is a permanent cell line capable of expressing isoproteins in culture. No other permanent cell line is known to express these isoproteins _in vitro_. The immunohistochemical staining of the GC-1 cell line using a monoclonal antibody against LDH-C and a monospecific rabbit polyclonal antibody to the testicular isoform of cytochrome c demonstrated the reactivity of the antibody to LDH-C and cytochrome c. The antibodies against LDH-C and cytochrome $c_t$ are described and identified in Goldberg et al., _Science_ 196:1010-1012 (1977), incorporated herein by reference.

The designation of primary spermatocyte usually implies that the cell in question has stopped dividing by mitosis, has differentiated and is entering the 1st meiotic division. Both the LDH-C and cytochrome $c_t$ gene products are known to start being expressed at the preleptotene/leptotene transition. By immortalizing the germ cell line at this stage, the large T antigen expression (a nuclear factor that normally regulates SV40 viral replication) has "locked" the cells at the developmental, and gene expression, stage of early meiosis while forcing the cells to cycle again by mitosis resulting in the proliferation of the cells and continued expression of the isoproteins.

## EXAMPLE IV
## Formation of In Vitro Tubule-like Structures

One characteristic of the _in vitro_ immortalized cell lines of the present invention is their ability to associate and reconstitute seminiferous tubule-like structures when plated together. The primary spermatocyte line GC-1 appears in the center of these

formations while Sertoli, peritubular and Leydig cells contribute to stablishing the borders betwe n the g rm c ll cores.

At least one of the somatic cell components is labeled with a fluorescent marker to facilitate following the distribution of the individual somatic cell components. A lipophilic carbocyanine membrane probe, DiI (D-282), was used to label the somatic cell lines. The fluorescent dye, DiO (D-275), can also be used to label the cell lines. The cells were incubated for 1 hour at 37°C in serum-free D-MEM containing 10 μg/ml DiI. The cells were subsequently washed twice in PBS prior to the co-culture experiments. The in vitro structures produced by non-tumorigenic, freshly isolated testicular cells were similar to those produced by the immortalized cell lines.

## EXAMPLE V
## Co-Transfected Cell Lines

Primary mouse germ cells, obtained as described below, were cotransfected plasmid pSV3-neo (ATCC NO. 37150), containing the LTAg gene and the $neo^r$ gene, and the plasmid LTRp53cG9, containing the gene encoding for temperature sensitive [$val^{135}$]p53 (described in Fukasawa et al. Mol and Cell Biol 11: 3472 -3483 (1991)), incorporated herein by reference. The ($val^{135}$)p53 is non-functional at 39°C, and functional at 37°C and 32°C. As described in detail below, at the non-permissive temperature of 39°C, the co-transfected cell lines continued to undergo mitosis because the newly introduced p53 gene was inactive and LTAg retained its immortalizing ability by binding to and inactivating the endogenous wtp53. At the permissive temperatures of 37°C and 32°C, excess p53 bound to LTAg and reduce or abolish its immortalizing properties.

### A. Obtaining Cell Lines

A single cell suspension was btained from decapsulated testes of sexually mature Balb/c mice as previously described above in Example I above. A cell fraction enriched with spermatocytes was then isolated using the STA-PUT™ (Johns Scientific, Toronto, Canada) gravity sedimentation procedure at 4°C according to the procedure described in Romrell et al, Dev. Biol. 49: 119-131 (1976), and Peden et al, Virol 168, 13 (1989), each incorporated herein by reference. Spermatocytes were cultivated in 60 mm tissue culture dishes (Falcon) and completed CMRL-1066 medium until a monolayer was formed.

Cotransfection was performed with two plasmids, the pSV3-neo plasmid which contains the LTAg gene and the $neo^r$ gene, and the LTRp53cG9 plasmid which contains the temperature sensitive [$val^{135}$]p53gene, and which was previously described in Michalowitz et al. Cell 62, 671-680 (1990) and Martinez et al. Genes & Develop. 5, 151 (1990), each incorporated herein by reference, and was provided by Dr. Channing Der, University of North Carolina, Chapel Hill, North Carolina. Cotransfection of 12.5$\mu$g pSV3-neo (ATCC #37150) and 12.5$\mu$g LTRp53cG9 per dish was performed by the calcium phosphate method described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989), incorporated herein by reference. G418 (Geneticin, Gibco, Chagrin Falls, OH), at a concentration of 200 $\mu$g/ml of active substance, was used for the selection of neomycin resistant colonies.

After selection with G418, two cell clones, designated GC-2spd(ts) and GC-3spd(ts) were obtained and cultivated after crisis for a period of six months.

B. Characterizing the Cell Lines

The two cells lines GC-2spd(ts) and GC-3spd(ts) showed adherence in tissue culture and were contact inhibited. No clonal proliferation was observed on soft agar cultures, indicating that these cells are immortalized but not transformed.

The cells lines were characterized according to morphology, growth characteristics, and immunocytochemistry at various temperatures. To determine the presence and location of LDH-C and cytochrome c, immunocytochemical analysis was performed as follows. The cells were grown at 37°C and 32°C to monolayers on LABTEK slide chambers (Nunc, Inc., Naperville, IL) in D-MEM completed medium (as described in Hofmann et al., Exp Cell Res 201, 417 to 435 (1992)), fixed with cold methanol, washed in PBS and incubated in PBS at 4°C for at least 2 days. Monolayers were stained using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to Example IIIC above using a polyclonal antibody against the testis-specific LDH-C isozyme, and rabbit polyclonal antibodies against the testis specific isoform of cytochrome c (cytochrome $c_t$), both supplied by Dr. Erwin Goldberg (Northwestern University, Illinois), and counterstaining with hematoxylin, fast green or neutral red. p53 was detected with a monoclonal antibody against mouse wt53 (Ab-1, clone 421, Oncogene Science, Manhasset, NY).

C. Immunocytochemical Characteristics of Cell Lines at Different Temperatures

39°C: As seen under phase contrast microscopy at 39°C, the GC-2spd(ts) and GC-3spd(ts) cell lines were large and spherical, and grew rapidly with a doubling time of 18 hours. Specific markers for meiotic germ

cells, LDH-C and cytochrome $c_t$, were express d at low l vels. p53 overexpression was confined to the cytoplasm.

<u>37°C</u>: At 37°C, both cell lines grew more slowly, with a doubling time of 24 hours. Figure 1 shows staining patterns for p53, LDH-C, and cytochrome $c_t$ for GC-2spd(ts) and GC-3spd(ts) at 37°C (Figure 1A) and 32°C (Figure 1B). At 37°C, LDH-C expression was enhanced, but cytochrome $c_t$ was not identifiable by immunocytochemistry, as shown in Figure 1A. The p53 protein was expressed in both the cytoplasm and the nucleus in both cell lines.

At 37°C, groups of cells showed signs of morphologic differentiation already visible at the light microscopy level. In some cells of both lines, but particularly in GC-2spd(ts), a dark granule appears at one pole of the nucleus, whereas the cell cytoplasm at the other pole of the nucleus become elongated.

Morphological differentiation of GC-2spd(ts) was examined by growing the cell lines to confluency in a LabTek chamber and fixed with cold methanol. Periodic acid-Schiff (PAS) staining was performed according to the methods described in Sheenan et al. <u>Theory and Practice of Histotechnology</u>. Battelle Press, Columbus, OH (1987). Immunocytochemical staining was performed as described above (Example III), using a monoclonal antibody, HS-63 (donated by Erv Goldberg, Northwestern University, Evanston, IL), against the sperm acrosome antigen MSA-63 (as described in Liu et al, <u>Biol Repr 46</u>, 937 to 948 (1992). For electronmicroscopy, cell monolayers were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, postfixed in $OsO_4$ and embedded in an epoxy resin by usual methods. Ultrathin sections were stained with uranylacetate and lead citrate, and visualized with a JEOL 100 cx electron microscope.

Electomicroscopic analysis confirmed the pr senc of this structure, which is shown in th el ctron micrograph of Figure 2C, and identified it as the developing sperm acrosome. Figure 2 shows morphological differentiation of the line GC-2spd(ts). Figure 2A shows periodic acid-Schiff (PAS) staining of the acrosomal granule in a round spermatid. This granule is membrane-bound and appeared to be derived from the Golgi apparatus. As expected, this structure shows periodic acid-Schiff's staining (performed as described in Hess, R.A. Biol. Repr 43, 525-542 (1992)), as is shown in Figure 2A and is positive immunocytochemically with a monoclonal antibody against a sperm acrosome antigen, MSA-63, as is shown in Figure 2B. Figurd 2C shows an electron micrograph of the acrosomel granule.

Flow cytometry analysis was performed on the GC-2spd(ts) cell line grown at 37°C for several generations as follows. Cells were cultivated to confluency in 25 $cm^2$ tissue culture flasks (Falcon), typsinized, fixed in cold methanol, and RNA degraded with 100 $\mu$g/ml RNase. Cell nuclei were then stained with 50 $\mu$g/ml propidium iodide and fluorescence recorded with a EPICS Profile flow cytometer (Coulter). Cell cycle data were analyzed with Multicycle software (Phoenix Flow Systems).

Flow cytometry analysis of the GC-2spd(ts) cell line performed as described above showed that at 37°C the cells first exhibited a DNA content which is diploid (G1) and tetraploid (G2), corresponding to a diploid cell cycle. Figure 3A shows the 16th generation of cell in culture, showing a diploid cell cycle only. However, prolonged cultivation of these cells at 37°C (over 20 generations) resulted in the appearance of a third and haploid peak, with flattening of the G2 values (Figures 3B and 3C). Figure 3B shows the 24th generation of the

same cells, which show a diploid cell cycle, as well as an extra haploid peak. Figur 3C shows the 30th generation of the sam cells, which shows an increase of the percentage of haploid cells. Figure 3D shows control mouse testis cells.

Haploidy was shown only at 37°C, indicating that the cell cycle has to be maintained in order for the cells to complete the second meiotic division. It appeared that certain cells belonging to a pool of continually dividing secondary spermatocytes were able to proceed through the second meiotic division and to differentiate into spermatids. This phenomenon was attributed to the behavior of the p53 protein at 37°C in this cell line. The borderline temperature of 37° seemed to allow the expression of both mutated and wt p53.

In contrast, the GC-3spd(ts) cell line did not progress into meiosis. In this line, p53 was expressed almost exclusively in the cytoplasm (mutant form) at 37°C, and only in the nucleus (wt form) at 32°C. Since at this temperature the cell cycle is blocked, the cells were probably prevented from undergoing the second meiotic division.

<u>32°C</u>

At 32°C, in both lines, cell growth was slowed progressively and the cells died after an average of 10 generations. This was attributed to the expression of the wild-type exogenous p53 gene that, in excess, blocked the immortalizating action of LTAg and arrested the cell cycle. In these cells, p53 was expressed almost exclusively in the nucleus. LDH-C and cytochrome ct expression were markedly enhanced as is seen in Figure 1. The germ cell line bearing only the LTAg, GC-1, continued to proliferate at 32°C and maintained the same morphological features, whereas GC-2spd(ts) and GC-

3spd(ts) cells become spindle-like, exhibited a long cytoplasmic prolongation and a much smaller cell body. The number of cells bearing the acrosomic granule increase from 3% at 37°C to 10% at 32°C. Moreover, by cultivating these cells at 32°C in serum-free medium, at least 30% of the cells produced the acrosomic granule. Cells that were switched very early from 37°C to 32°C never underwent meiosis but continued to differentiate for 10 generations, whereas GC-2spd(ts) cells that underwent meiosis at 37°C and were switched to 32°C died very quickly, leaving intact the pool of proliferating cells.

Therefore, by using the immortalizing ability of LTAg under the conditional modulation of tsp53, cell line GC-2spd(ts) was obtained, capable of serving as a stem cell pool of spermiogenic cells. These cells underwent meoisis _in vitro_ and produced round and elongated spermatids. This cell line provides a unique system to study meiosis and germ cell differentiation _in vitro_.

Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

We claim:

1. An immortalized germ cell line.

2. The immortalized germ cell line of claim 1, wherein said germ cell line is of murine origin.

3. The immortalized germ cell line of claim 1, wherein said germ cell line is a spermatogonia, primary spermatocyte, secondary spermatocyte or spermatid.

4. The immortalized germ cell line of claim 3, wherein said germ cell line expresses a testicular isoform of cytochrome c and LDH-C.

5. The immortalized germ cell line of claim 1, wherein said germ cell line is GC-1.

6. A method for producing an immortalized non-tumorigenic germ cell line comprising:

(a) growing a non-tumorigenic germ cell in the presence of non-tumorigenic Sertoli, peritubular and Leydig cells; and

(b) immortalizing said germ cell with an immortalizing molecule.

7. The method of claim 6, wherein said immortalizing molecule is a large T antigen.

8. The method of claim 7, wherein said large T antigen is an SV40 large T antigen.

9. The method of claim 6, wherein said immortalizing molecule is transfected into the genome of said germ cell to immortalize said germ cell.

10. A method of producing a testicular isoform of cytochrome c *in vitro*, comprising:

(a) culturing the immortalized germ cell line of claim 4 *in vitro*; and

(b) harvesting said cytochrome c.

11. A method of producing a testicular isoform of LDH-C *in vitro*, comprising:

(a) culturing the immortalized germ cell line of claim 4 *in vitro*; and

(b) harvesting said LDH-C.

12. An immortalized somatic cell line derived from a non-tumorigenic mammalian testicular cell capable of being cultured *in vitro* for at least one month.

13. The immortalized cell line of claim 11, wherein said somatic cell line is a Sertoli, a peritubular or a Leydig cell line.

14. A method for culturing a non-tumorigenic germ cell *in vitro* comprising contacting said germ cell with immortalized Sertoli, peritubular and Leydig cells.

15. A method for culturing an immortalized germ cell *in vitro* comprising contacting said immortalized germ cell with non-tumorigenic Sertoli, peritubular and Leydig cells.

16. The method of claim 14, wherein said immortalized germ cell is from the germ cell line GC-1.

17. A conditionally immortalized germ cell line capable of undergoing meiosis and differentiation <u>in vitro</u>.

18. The conditionally immortalized germ cell line of claim 16 wherein the germ cell line is a murin testicular germ cell line.

19. The conditionally immortalized testicular germ cell line of claim 17 wherein the germ cell line is secondary spermatocyte.

20. The conditionally immortalized germ cell line of claim 17 wherein the germ cell line comprises a first foreign gene encoding an immortalizing molecule and a second foreign gene encoding a temperature sensitive molecule which inactivates the immortalizing molecule at a temperature of 37°C or less.

21. The conditionally immortalized germ cell line of claim 19 wherein the first foreign gene encodes the large T antigen.

22. The conditionally immortalized germ cell line of claim 19 wherein the second foreign gene encodes a molecule which binds to the immortalizing molecule is an antiproliferative protein capable of binding to the immortalizing molecule inside the cell.

23. The conditionally immortalized germ cell line of claim 21 wherein the antiproliferative protein is p53.

24. The conditionally immortalized germ cell line of claim 22 wherein the cell line is GC-2spd(ts) or GC-3spd(ts).

25. A method for preparing an immortalized g rm c ll line, comprising integrating a gene encoding an immortalizing molecule into a germ cell.

26. The method of claim 24, wherein the gene encodes for the large T antigen.

27. A method for preparing a conditionally immortalized germ cell line comprising co-integrating a gene encoding an immortalizing molecule and a gene encoding molecule which inactivates the immortalizing molecule inside the transformed cell under certain conditions.

28. The method of claim 26 wherein the immortalizing molecule is a large T antigen.

29. The method of claim 26 wherein the inactivating molecule is a temperature sensitive antiproliferation molecule.

30. The method of claim 28 wherein the antiproliferative molecule is temperature-sensitive p53.

31. A method of producing a testicular isoform of lactate dehydrogenase in vitro, comprising:

(a) culturing the germ cell line of claim 19 at a temperature of 37°C or less; and

(b) harvesting said lactate dehydrogenase.

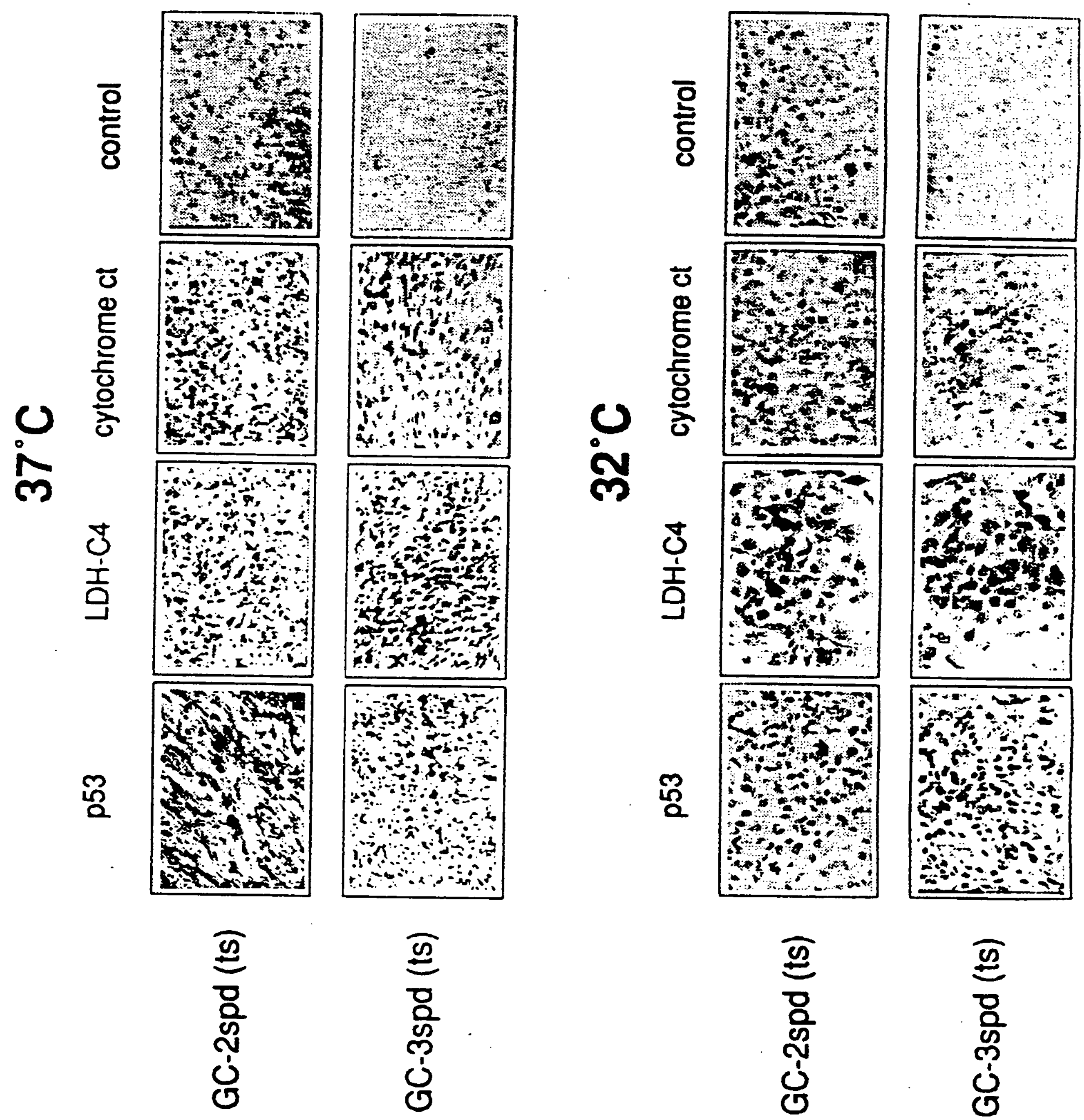
37°C
p53
LDH-C4
cytochrome ct
control
GC-2spd (ts)
GC-3spd (ts)
32°C
p53
LDH-C4
cytochrome ct
control
GC-2spd (ts)
GC-3spd (ts)

FIG. 1A

FIG. 1B

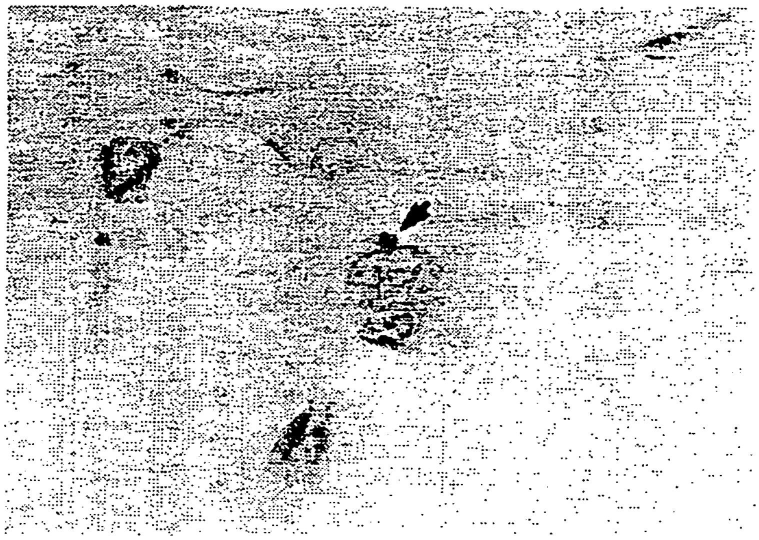

FIG. 2A

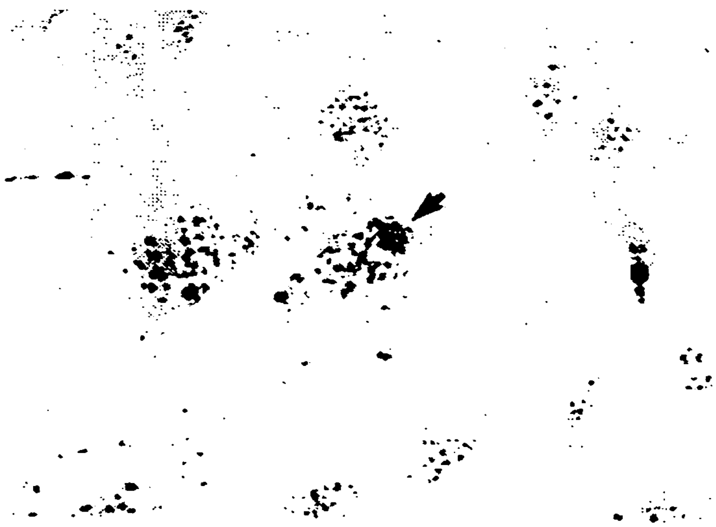

FIG. 2B

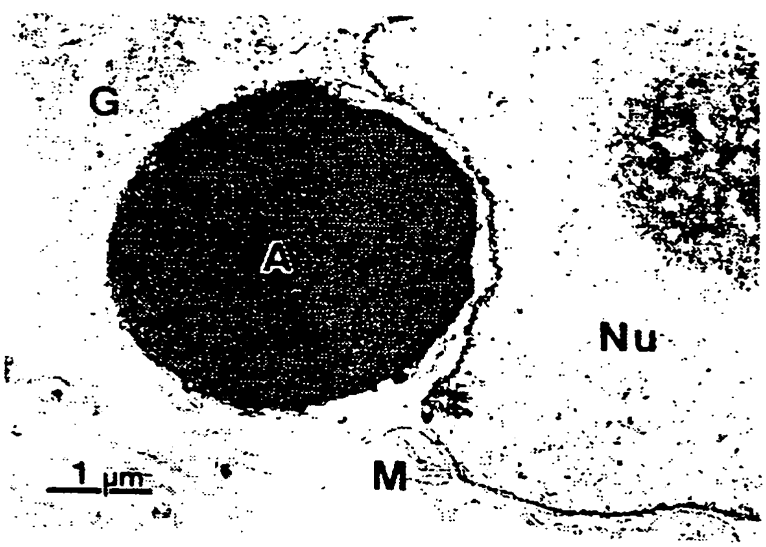


FIG. 2C

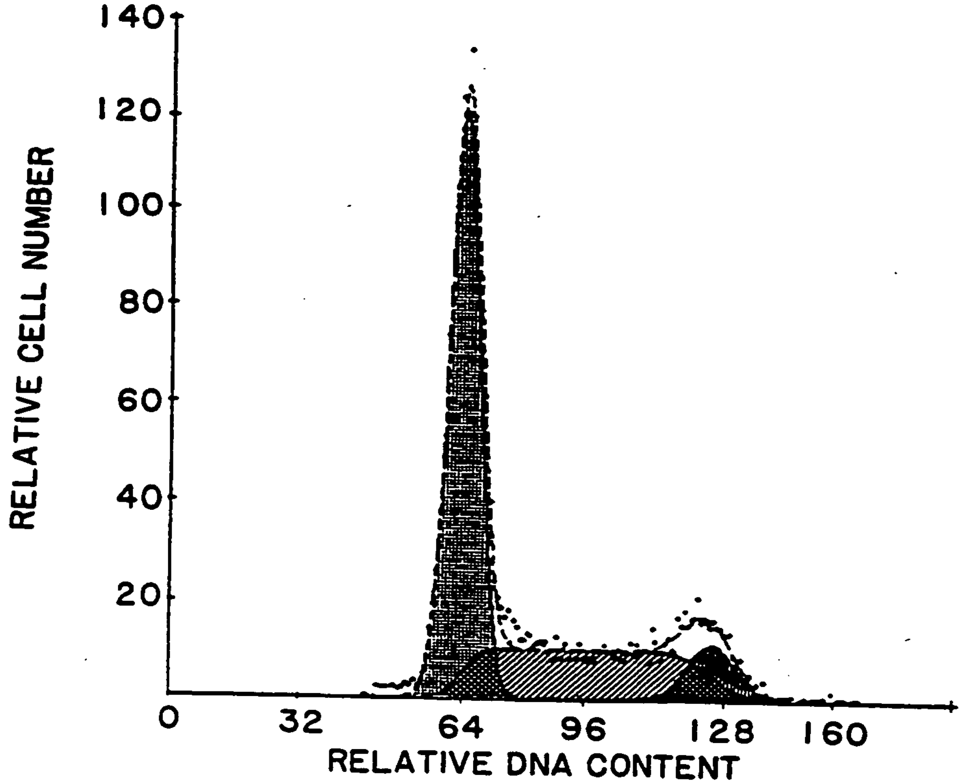


FIG. 3A

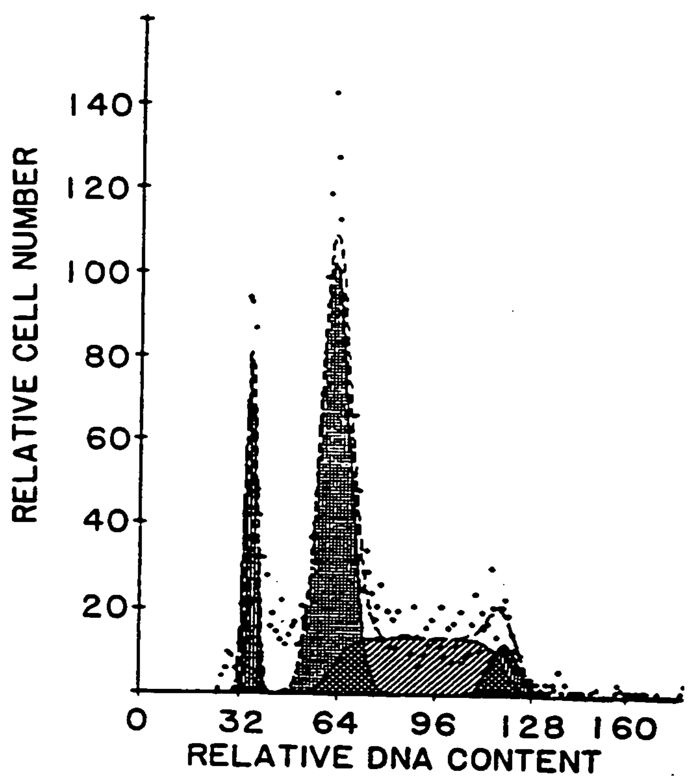


FIG. 3B

SUBSTITUTE SHEET (RULE 26)

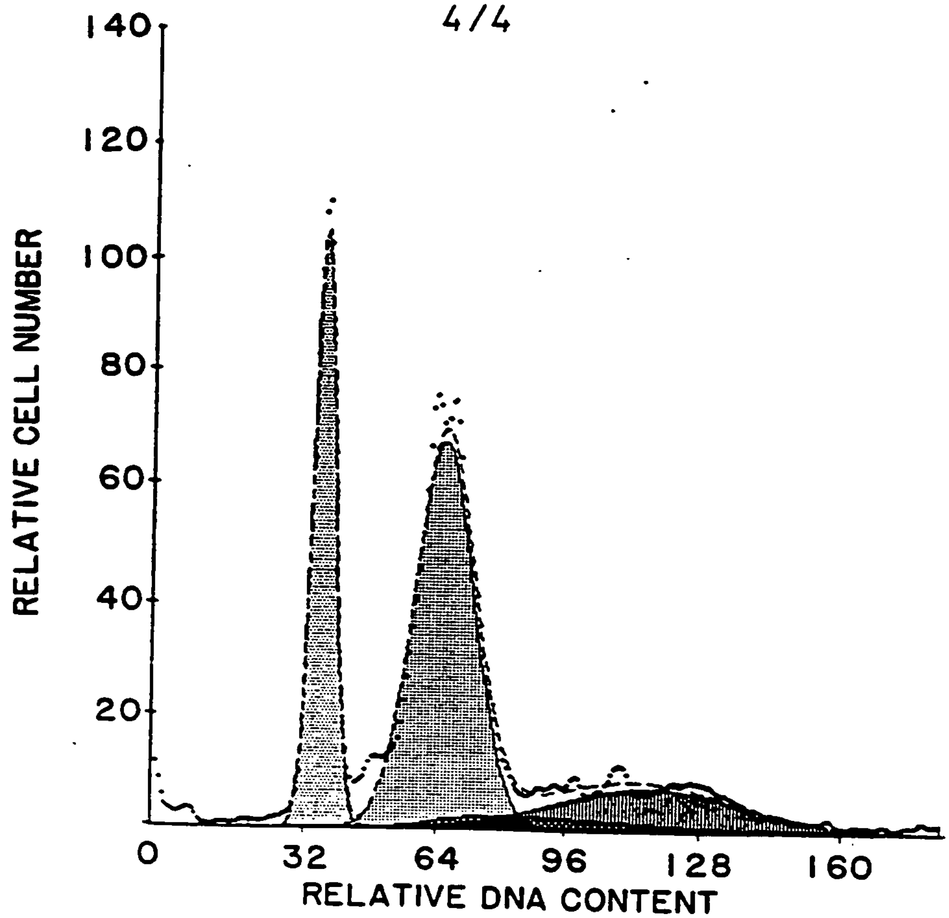
140
120
100
80
60
40
20
RELATIVE CELL NUMBER
0
32
64
96
128
160
RELATIVE DNA CONTENT

FIG. 3C

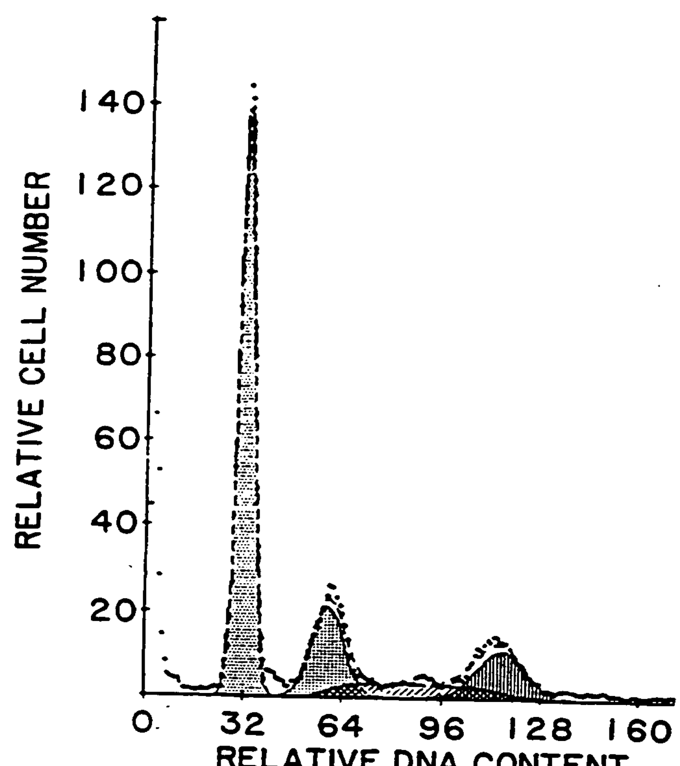
140
120
100
80
60
40
20
RELATIVE CELL NUMBER
0
32
64
96
128
160
RELATIVE DNA CONTENT

FIG. 3D

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/07654

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC(6) :C12N 5/10, 15/87
US CL :435/240.2, 172.3
According to Internati nal Patent Classification (IPC) r to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/240. 72.3

Documentation searched other than minimum documentation to the extent that such documents are included in the ields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, MEDLINE EXPRESS, BIOSIS, WPI
search terms: testicular, testes, germ cell

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>---<br>Y | Biology of Reproduction, Volume 23, issued 1980, J. P. Mather, "Establishment and Characterization of Two Distinct Mouse Testicular Epithelial Cell Lines", pages 243-252, entire document. | 1-4<br>----<br>5-31 |
| X<br>---<br>Y | Annals of the New York Academy of Sciences, Volume 383, issued 1982, J. P. Mather et al., "CULTURE OF TESTICULAR CELLS IN HORMONE-SUPPLEMENTED SERUM-FREE MEDIUM", pages 44-68, entire document. | 1-4<br>----<br>5-31 |
| X<br>---<br>Y | Journal of Ultrastructure Research, Volume 87, issued 1984, J. P. Mather et al., "Establishment of a Peritubular Myoid-like Cell Line and Interactions between Established Testicular Cell Lines in Culture", pages 263-274, entire document. | 1-4<br>----<br>5-31 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
26 OCTOBER 1994

Date of mailing of the international search report
03 NOV 1994

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>—<br>Y | Science, Volume 154, issued 02 December 1966, Y. Yasumura et al., "Establishment of Four Functional, Clonal Strains of Animal Cells in Culture", pages 1186-1189, entire document. | 1-4<br>—<br>5-31 |
| X<br>—<br>Y | Archives of Virology, Volume 115, issued 1990, C. S. Choi et al., "Establishment of transformed swine fibroblast cell lines using SV40 large T antigen", pages 227-237, entire document. | 1-9<br>—<br>10-31 |
| Y | Proceedings of the National Academy of Sciences (USA), Volume 75, No. 3, issued March 1978, J. Y. Chou, "Human placental cells transformed by tsA mutants of simian virus 40: A model system for the study of placental functions", pages 1409-1413, entire document. | 17-22, 24-29, 31 |